Combination therapy utilizing shRNA knockdown and an optimized resistant transgene for rescue of diseases caused by misfolded proteins

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Molecular knockdown of disease proteins and restoration of wild-type activity represent a promising but challenging strategy for the treatment of diseases that result from the accumulation of misfolded proteins (i.e., Huntington disease, amyotrophic lateral sclerosis, and α-1 antitrypsin deficiency). In this study we used alpha-1 antitrypsin (AAT) deficiency with the piZZ mutant phenotype as a model system to evaluate the efficiency of gene-delivery approaches that both silence the piZZ transcript (e.g., shRNA) and restore circulating wild-type AAT expression from resistant codon-optimized AAT (AAT-opt) transgene cassette using adeno-associated virus (AAV) vector delivery. After systemic injection of a self-complimentary AAV serotype 8 (scAAV8) vector encoding shRNA in piZZ transgenic mice, both mutant AAT mRNA in the liver and defected serum protein level were inhibited by 95%, whereas liver pathology, as monitored by dPAS and fibrosis staining, reversed. To restore blood AAT levels in AAV8/shRNA-treated mice, several strategies to restore functional AAT levels were tested, including using AAV AAT-opt transgene cassettes targeted to muscle and liver, or combination vectors carrying piZZ shRNA and AAT-opt transgenes separately, or a single bicistronic AAV vector. With these molecular approaches, we observed over 90% knockdown of mutant AAT with a 13- to 30-fold increase of circulating wild-type AAT protein from the shRNA-resistant AAT-opt cassette. The molecular approaches applied in this study can simultaneously prevent liver pathology and restore blood AAT concentration in AAT deficiencies. Based on these observations, similar gene-therapy strategies could be considered for any diseases caused by accumulation of misfolded proteins.

neurodegenerative disease | codon modification | mutant protein | therapeutic | dPAS staining

A number of diseases are caused by misfolded proteins (e.g., cycstic fibrosis). In addition, it is well known that misfolded proteins accumulate intracellularly and cause cell damage, leading to diseases. For example, superoxide dismutase 1 mutations result in protein aggregation that leads to amyotrophic lateral sclerosis, Alzheimer's disease, and Huntington disease are related to mutant amyloid-β (Aβ) and huntingtin (Htt) protein accumulations, respectively (1). Clearance of these mutant proteins by drugs has resulted in improvement of the clinical outcome. To rescue the complete phenotype, restoration of normal protein gene therapy is currently being evaluated. In this study, we used α-1 antitrypsin (AAT) deficiency with the piZZ phenotype (glu 342 → lys: GAG-AAG) as a disease model to evaluate the efficiency of silencing the piZZ transcript and restoring circulating AAT levels using adeno-associated virus (AAV) vector-mediated gene delivery.

AAT deficiency with the piZZ mutation results in severe liver disease and lung damage caused by misfolded protein accumulation in the endoplasmic reticulum of the liver cells and low levels of AAT in circulation, respectively. Currently, liver transplantation is the only treatment able to cure the disease. Gene therapy is an attractive alternative for AAT deficiency because of the well-defined therapeutic endpoint and its wide therapeutic index. Several vectors have been shown to express detectable serum levels of hAAT in animal models. AAV vectors have the advantage of high in vivo transgene expression, low toxicity, and the capacity to express its transgene in both mitotically active and quiescent cells. These characteristics of AAV are especially suited for the transfer and persistent expression of the human AAT gene (2). Clinical trials with AAV/hAAT have been performed (3, 4). AAV2 transduction in the liver can initiate very high transgene expression with a fivefold increase in AAT expression relative to that from muscle (5). Current mechanisms for rAAV transduction involve the conversion of a single-stranded (ss) to a double-stranded (ds) genome. To eliminate the necessary ss- to ds-genome conversion, we have recently developed a unique form of AAV vector that packages a ds template (dsAAV), also called a self-complementary (sc) genome (6). These vectors are not subject to the classic "slow onset" of gene expression typically observed with traditional ssAAV vectors. Recently, 12 serotypes of AAV have been isolated and used as gene-delivery vehicles, with over 100 additional variants described. The capsid sequences of AAV serotypes vary in their homology to type 2. Heterogeneity is found predominantly within the exposed regions on the capsid surface of different serotypes, suggesting serotypes may have distinct mechanisms of transduction. To date, different serotypes of AAV vectors and transgene cassettes have been used to improve liver transduction; serotypes 1, 6, 8, and 9 showed improved transduction over AAV2, with AAV8 being the best (7).

Lung disease associated with AAT deficiency can be prevented by replenishment of the deficient gene product via gene therapy. However, this strategy does not prevent the liver disease caused by intracellular accumulation of the misfolded proteins. Strategies to prevent liver disease should focus on correction of the mutant gene at the DNA or mRNA level, or suppression of mutant RNA expression. A few practical methods have been tested to repair or shut down the expression of mutant genes, including antisense oligomers, chimeric RNA:DNA oligomers, and ribozymes. Post-transcriptional silencing by siRNA has emerged as a powerful tool for genetic analyses in mammalian cells and has the potential to produce novel therapeutics. Unfortunately, oligonucleotide-mediated siRNA transfer suffers from several limitations, such as high cost, nonspecific toxicity of transfection, and transient suppression of gene expression. To overcome these problems, several groups have described alternative systems for siRNA expression within mammalian cells that rely on DNA vectors. In general,

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these DNA vectors use an RNA polymerase III (Pol III) promoter to express short dsRNA in the form of an inverted repeat sequence containing a hairpin loop. Such small hairpin RNAs (shRNA) are efficiently processed into siRNAs inside the cells and achieved sequence-specific gene silencing. AAV vectors have been used to deliver shRNA for a variety of purposes. Here we used an AAV vector to package an AAT-targeting shRNA to knock down piZZ transcripts in the liver. Decreased amounts of piZZ protein in the liver cells may delay liver disease development or progression. Alone, piZZ knockdown cannot resolve other complications caused by AAT deficiency, but supplementation of shRNA-resistant wild-type AAT may solve the problem.

In the present study, we have shown that AAV vectors encoding a single AAT shRNA are effective in decreasing human AAT expression in cell lines and in transgenic mice with the piZZ phenotype. Muscular administration of an AAV6/AAT vector restored AAT expression in the blood. Utilization of an optimized AAT construct (AAT-opt) delivered by an AAV vector not only induced higher transgene expression than wild-type AAT cDNA, but also escaped the degradation of wild-type AAT-targeted shRNA when AAT-opt and shRNA were simultaneously delivered into the liver via either a single vector or dual vectors.

Results

Silencing Effect of shRNA on AAT. siRNA has been widely used to knock down target gene expression for a variety of purposes. We have designed three siRNAs that have the potential to silence AAT expression (Table S1). To test the siRNA silencing effect, we have established the cell lines 293/wild-type AAT and 293/piZZ. After transfection of three candidate piZZ/siRNA oligos into these cells, AAT in the supernatant was measured at 48 h. All three piZZ/siRNA oligos exerted AAT gene silencing, with over 70% efficiency in both 293/wild-type AAT and 293/piZZ cells (Fig. S1*A*). These three siRNAs were individually cloned into scAAV constructs, which were encapsidated into the AAV serotype-2 virion. After transduction of 293/wild-type AAT or 293/piZZ with AAV2/shRNA vectors, the shRNA2 construct exerted a better silencing effect than the other two shRNAs (Fig. S1*B*); thus, shRNA2 was used for all in vivo studies.

To examine whether the AAV/shRNA2 vector promoted AAT gene silencing in the mouse liver, we packaged an AAT shRNA2 construct into the AAV8 capsid, because AAV8 has been shown to transduce the mouse liver with almost 100% efficiency with 6×10^{10} particles of double-stranded GFP virus (Fig. S2). We coadministered 6×10^{10} particles of AAV8/shRNA2 and 1×10^{11} particles of AAV2/CBA-AAT viruses into wild-type BALB/c mice via tail-vein injection. As shown in Fig. S3*A*, the coadministration of AAV8/shRNA significantly inhibited AAT expression in blood compared with coadministration of AAV8/scramble shRNA or control with only AAV2/CBA-AAT injection ($P < 0.05$). There was no significant difference in AAT expression between the latter two groups ($P > 0.05$). We also detected liver function after AAV8/shRNA administration to assess possible liver toxicity from the shRNA, and no significant change was observed between the shRNA group and controls (Fig. S3 *B* and *C*).

Knockdown of piZZ-AAT Transgene Expression with shRNA. Next we tested whether this shRNA construct could similarly inhibit piZZ-AAT expression in piZZ-transgenic mice. At week 2 after tail-vein injection of AAV8/shRNA2, compared with scramble shRNA-treated mice and control without any treatment, the AAT-shRNA construct led to over 95% suppression of AAT expression ($P < 0.05$) (Fig. 1 *A* and *B*). We also measured the AAT transcript in the liver with shRNA2 therapy, and consistent with the AAT levels in the blood, AAT mRNA copy numbers in the liver were over 100-fold lower than control and scramble shRNA mice (Fig. 1*C*). To examine whether shRNA treatment could improve liver pathology, which is associated with misfolded mutant piZZ AAT protein accumulation and fibrosis in the liver, we performed dPAS and Sirius Red staining of liver sections to detect mutant piZZ-AAT inclusion in liver cells and fibrosis in the liver, respectively. The liver sections were visualized by microscopy and processed by ImageJ software (Fig. S4). As shown in Fig. 1, the accumulation of mutant piZZ-AAT protein was significantly reduced in AAV8/shRNA2-treated mice compared with mice with scramble shRNA (Fig. 1 *D* and *E*). Similarly, liver fibrosis was reversed in mice with shRNA2 therapy (Fig. 1 *D* and *F*). It is interesting to note that liver transaminase levels were either not affected or slightly increased to levels similar to those seen in nontreated control mice and with AAV8/scramble after AAV application. However, AAV8/shRNA2-treated piZZ mice showed a decline of both alanine aminotransferase (ALT) and aspartate aminotransferase (AST) liver enzymes (Fig. 1 *G* and *H*). The silencing effect of shRNA on piZZ AAT expression was still observed after 9 wk (Fig. S5). The data imply that administration of AAV8/shRNA2 can improve liver function because of a shRNA-mediated decrease in piZZ protein accumulation.

Muscular Administration of AAV6/AAT Restored AAT Expression from shRNA Silencing. The AAT deficiency caused by the piZZ mutation results not only in liver disorders because of misfolded protein accumulation, but also lung diseases resulting from the lack of a functional gene product. shRNA knockdown can prevent liver disease development but has no effect on the lung disorders, as there is no replenishment of AAT deficiency in circulation. One solution to this problem would be to use two vectors: one for knockdown and the other for gene replacement. An important obstacle to avoid in combining knockdown and restoration is to specifically knock down the mutant transcript but avoid knockdown of the corrected transcript. To circumvent this problem, we initially targeted two different organs: liver for piZZ knockdown and muscle for wild-type AAT replacement. We chose to use AAV6 for intramuscular injection, as this AAV serotype is considered one of the best for initiating transgene expression in muscles (8). After injection of AAV8/shRNA2 vector systemically in piZZ mice, AAV6/AAT was injected into muscle. At week 2 after AAV6 administration, blood AAT levels were 10-fold higher in mice given the AAV6/AAT vector than in mice given only the AAV8/shRNA knockdown vector (Fig. 2). This result indicates that utilization of shRNA delivered by AAV can efficiently knock down piZZ expression and AAT expression can be restored from muscle transduced with an AAV vector encoding a wild-type AAT construct.

Optimized AAT Transcript Is Resistant to Wild-Type AAT shRNA. As explained above, same-cell gene knockdown and replacement can result in therapeutic gene degradation if the shRNA sequence matches the therapeutic transgene. One resolution for this obstacle is the targeting of distinct tissues for knockdown and gene replacement. However, in animal experiments, higher transduction using AAV vectors can be achieved by targeting liver than muscles; as such, knockdown and replacement events should ideally occur in the liver. To achieve such a goal, the replacement gene sequence would need to be altered in such a way as to render it unique from the shRNA-recognition sequence. Optimization of gene transcripts alter transgene sequences yet retain the translated amino acid sequence, and potentially increase the amount of gene product. In our prior study, utilization of an optimized coagulation factor 9 (F9) induced fivefold higher transgene expression than a wild-type F9 construct with AAV delivery (9). Based on this experience, we have generated an optimized AAT cDNA construct (pTR/CBA-AAT-opt) for expression specifically in mouse tissues. To test the ability of the AAT-opt construct to mediate enhanced transgene expression, pTR/CBA-AAT or pTR/CBA-AAT-opt was transfected into B16-F1 cells (mouse

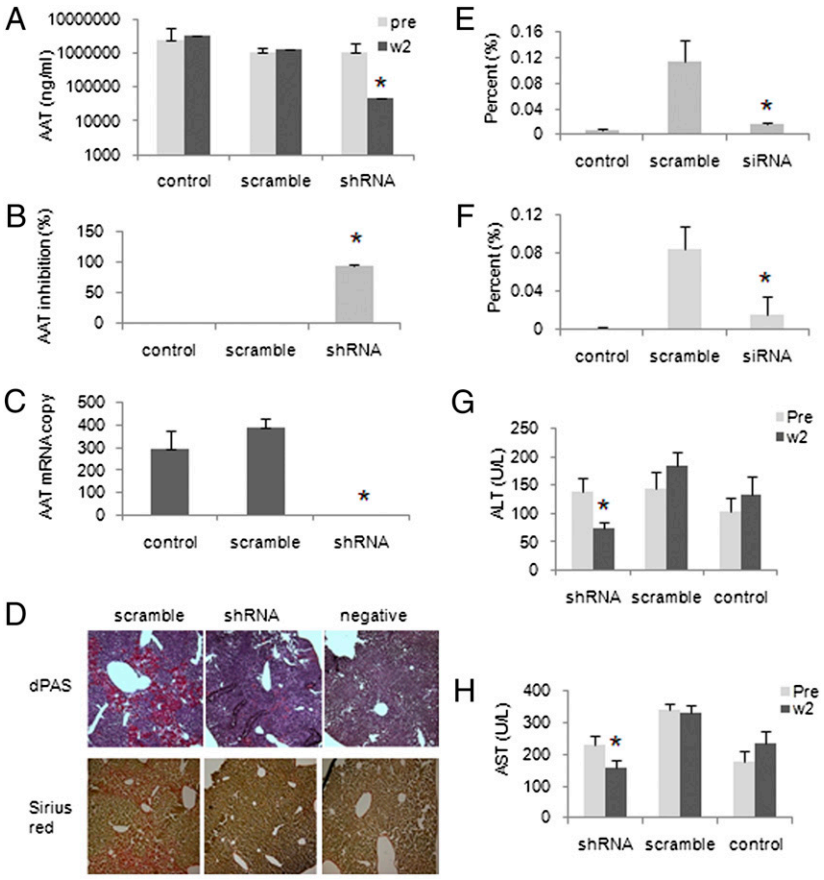

Fig. 1. The knock down efficiency of shRNA in piZZ mice. Female piZZ mice at the age of 6 to 8 wk were injected with 6×10^{10} particles of dsAAV8/shRNA2 via tail vein. At week 2 after AAV administration, blood and the liver were collected for evaluation of AAT expression and liver function. (*A*) AAT protein concentration in the blood. *$P < 0.05$ compared with pre-shRNA treatment. (*B*) Inhibition of shRNA on AAT protein production was calculated by (1 − the ratio of the AAT concentration in blood at week 2/the AAT level at preinjection) × 100%. *$P < 0.05$ compared with pre-shRNA treatment. (*C*) The expression of piZZ transcripts with shRNA treatment. The AAT mRNA copy number was calculated with normalization to β-actin. The results are the mean of four mice, and error bars indicate SD. *$P < 0.05$ compared with untreated or scramble shRNA-treated mice. (*D*) dPAS and Sirius Red staining of liver sections (original magnification: × 10). (*E* and *F*) The quantification of dPAS staining and Sirius Red staining, respectivly. *$P < 0.05$ compared with scramble shRNA treatment. (*G* and *H*) The measurement of liver transaminases ALT and AST, respectively. *$P < 0.05$ compared with pre-shRNA treatment.

melanoma cell line), and 24 h posttransfection the AAT levels were detected by ELISA in the culture supernatant. The optimized coding sequence resulted in a 30% increase in AAT secretion compared with wild-type AAT DNA (Fig. S6*A*). Interestingly, no difference in AAT expression (between AAT-opt and AAT) was observed in a human cell line (293 HEKs), perhaps because of the mouse-specific optimization algorithm applied. The optimized AAT construct was packaged into an AAV2 virion and injected into mice via the tail vein. Consistent with the in vitro result, AAV2/AAT-opt produced 30% to 50% more AAT in the blood than with the AAV2/AAT vector (Fig. S6*B*). These results further support that optimization of the AAV-packaged transgene can enhance trangene expression.

By alignment, there are key nucleotide mismatches between wild-type and optimized AAT sequences at the siRNA targeting location; specifically, four nucleotide mismatches were found for siRNA2 between optimized and wild-type AAT constructs. To test whether the expression of AAT from the optimized AAT cDNA cannot be inhibited by our used siRNAs, we transfected pCBA-AAT-opt or pCBA-AAT with each of three siRNAs (5 nM). The next day, we measured the AAT levels in the media. As shown in Fig. S7*A*, all three siRNAs knocked down wild-type AAT expression about 30% to 40%; however, there was no suppression of AAT-opt expression (Fig. S7*A*). To confirm the in vitro data in mice, coadministration of AAV8/shRNA2 and AAV2/CBA-AAT-opt into mice via tail-vein injection demonstrated no inhibition of AAT expression in the blood compared with or without scramble shRNA ($P > 0.05$) (Fig. S7*B*). These results suggest that AAT-opt can be used to escape mRNA degradation by wild-type AAT siRNAs and potentially restore AAT expression. Of note, no liver damage was observed with coadministration of AAV8/shRNA and AAV2/CBA-AAT-opt vectors (Fig. S7 *C* and *D*).

Coadministration of shRNA and Optimized AAT Delivered by Different Serotypes of AAV Rescued AAT Expression. We and others have previously shown that different AAV serotypes result in differential transduction dynamics and transgene expression in the liver. Specifically, AAV8 and -9 induce a better and faster gene expression than other serotypes. As AAV8 was chosen to deliver shRNA, we wondered whether utilization of AAV9 to deliver the AAT-opt construct into the liver could restore AAT expression in the blood, as the piZZ transcript was silenced by AAV8/shRNA. There is low cross-reactivity of neutralizing antibodies between AAV9 and -8 capsids, suggesting that AAV9 may transduce target cells using different receptors or coreceptors from AAV8 (7). To avoid the potential competition of AAV9 and -8 during coinjection, we administered the AAV9/AAT-opt vector into piZZ mice 24 h after administration of AAV8/shRNA. After injection of AAV9/AAT-opt vector, AAT level in

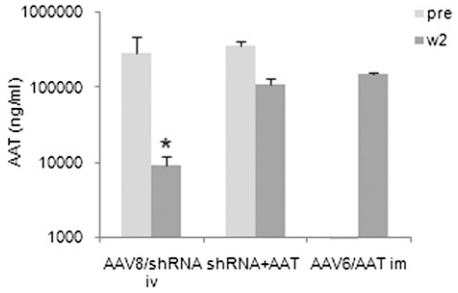

Fig. 2. The restoration of AAT expression from AAV intramuscular injection in PiZZ mice treated with shRNA. PiZZ mice were administered with 6×10^{10} particles of dsAAV8 vector via tail-veil injection and 1×10^{11} particles of AAV6/AAT via muscular injection, simultaneously. At week 2 after AAV administration, the blood was harvested and AAT expression was detected by ELISA. The results are the mean from four mice and error bars indicate the SD. AAV8/shRNA iv: piZZ mice with only treatment of AAV8/shRNA; shRNA +AAT: piZZ mice with the treatment of AAV8/shRNA via intravenous and AAV6/AAT via intramuscular injection; AAV6/AAT im: C57BL mice with intramuscular injection of AAV6/AAT vector. *$P < 0.05$ compared with pre-shRNA-treated mice.

the blood was measured 2-wk postinjection. In the shRNA-only group, compared with preinjection of AAV vector, over 95% inhibition of AAT expression was observed (Fig. 3*A*). However, the AAT level in the blood was similar between pre- and posttreatment in the group with shRNA plus AAV9/AAT-opt ($P > 0.05$) (Fig. 3*A*). Similar results were also seen at week 4 after AAV9/AAT-opt administration (Fig. S8*A*). We further collected the liver and measured the level of AAT mRNA at week 2 postinjection. With normalization to β-actin, about 100-fold lower wild-type AAT mRNA copy number was observed in shRNA-treated mice regardless of whether mice were treated with the AAV9/AAT-opt vector or left untreated ($P < 0.05$) (Fig. 3*B*). No mRNA of AAT-opt was detected in the untreated control and the shRNA-only treated mice, whereas 20 copies of AAT-opt mRNA were detected in mice with coadministration of shRNA and AAT-opt (Fig. 3*C*). These results further support that the optimized AAT construct can be used to escape shRNA-mediated degradation.

Restoration of AAT Expression and Silencing of piZZ in the Liver from a Single AAV Vector. Although different serotypes of AAV vectors may have very low neutralizing antibody cross-reactivity, and here we have successfully demonstrated the use of a dual-vector system to deliver shRNA and AAT-opt constructs separately into the liver, there remain a number of uncertainties regarding AAV biology, including serotype effects on AAV trafficking and endocytosis, which can impact the transduction efficiency of the later-administered AAV vector. Therefore, it could be that in some circumstances a single, dual-functioning vector would be ideal. To achieve this result, we cloned the shRNA and AAT-opt expression constructs into a single AAV cassette and studied the effect of an AAV8 vector delivering a single construct encoding shRNA2 and AAT-opt into the piZZ mouse liver, specifically on piZZ-AAT transcript silencing in the liver and restoration of normal circulating levels of AAT in the blood. Two weeks after tail-vein injection of AAV8/shRNA-AAT-opt, no decrease in blood AAT level was observed compared with that before injection (Fig. 4*A*). Moreover, no changes in blood AAT concentrations were observed 8 wk after injection of AAV8/shRNA-AAT-opt vector (Fig. S8*B*). As the ELISA is not specific for the mutant or wild-type AAT protein, this result could be interpreted as either successful knockdown combined with successful AAT-opt expression, or as the result of a nonfunctioning vector. To address this concern, we harvested the liver from AAV8-treated mice and measured the copy number of AAT transcripts

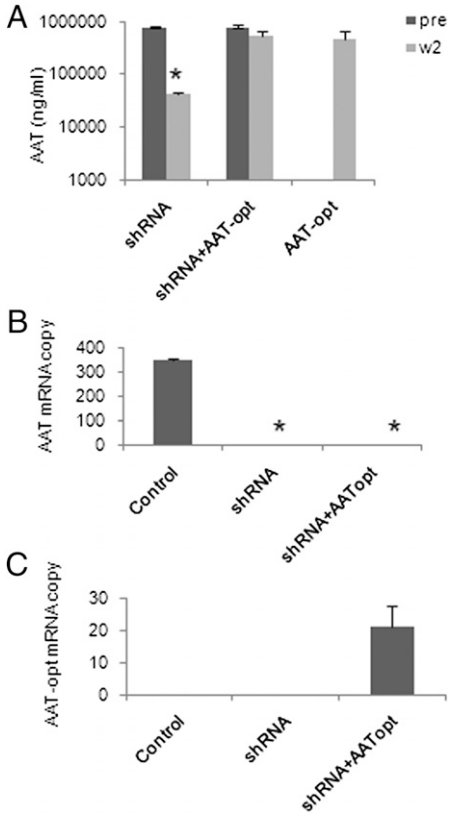

Fig. 3. Simultaneous knock down of piZZ and restoration of wild-type AAT in the blood, using two vectors in the liver of piZZ mice. The piZZ mice were receiving 6×10^{10} particles of dsAAV8/shRNA followed by administration of 2×10^{11} particles of AAV9/AAT-opt vectors via tail-vein injection on the next day. Two weeks later, the blood and the liver were collected for AAT expression (*A*), detection of the piZZ transcript (*B*), and the optimized AAT transcript (*C*). The results are the mean value from four mice and error bars indicate SD. shRNA: piZZ mice received only AAV8/shRNA2 vector via intravenous injection; shRNA+AAT-opt: piZZ mice with the treatment of AAV8/shRNA2 and AAV9/AAT-opt via intravenous injection; AAT-opt: C57BL mice were treated with only AAV9/AAT-opt via intravenous injection. *$P < 0.05$ compared with pre-shRNA-treated mice and control piZZ mice without treatment.

from the endogenous AAT-piZZ mutant allele and the delivered AAT-opt allele. As we observed with a two-vector approach described above, the AAT transcript copy number was remarkably decreased in mice with an AAV8/shRNA-AAT-opt injection compared with control mice ($P < 0.05$) (Fig. 4*B*). Importantly, a much higher copy number of AAT-opt transcript was observed in AAV8/shRNA-AAT-opt treated mice than mice receiving scAAV8/shRNA plus AAV9/AAT-opt (323.5 vs. 21.2, respectively) (Figs. 4*C* and 3*C*, respectively). These data indicate that delivery of shRNA and AAT-opt into the liver using a single AAV8 vector can efficiently knock down the piZZ transcript in the liver and restore normal AAT levels in the blood.

Discussion

In this study, we have demonstrated that a single siRNA2 was effective at inhibiting AAT expression. Utilization of AAV vector to deliver this shRNA2 also efficiently silenced AAT expression in vitro and in vivo. Over 95% knockdown of mutant AAT expression in the blood was achieved by delivery of AAV8/shRNA2 into the liver of piZZ mice, an effect similar to the degree of piZZ mRNA knockdown. Importantly, liver pathology was remarkably improved. After liver delivery of AAV8/shRNA2 to knockdown piZZ, intramuscular injection of AAV6/AAT vector was able to

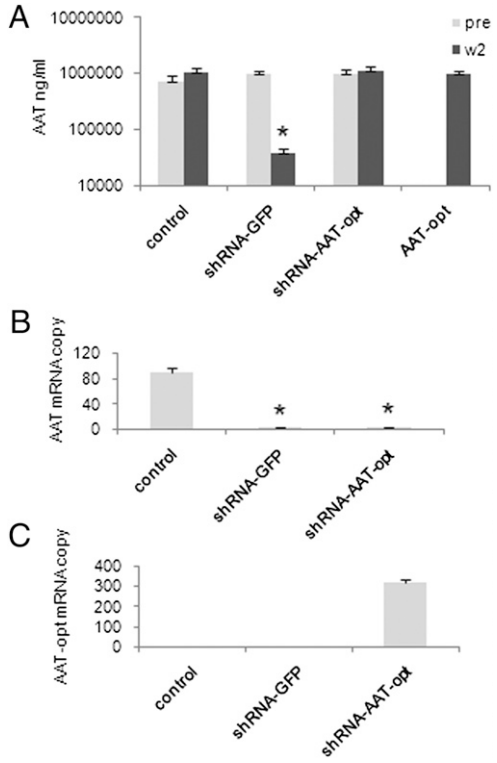

Fig. 4. Blood AAT restoration from the liver transduced with a single vector to deliver shRNA and optimized AAT. The piZZ mice were administrated with 3×10^{11} particles of AAV8/shRNA-AAT/opt vectors (which encode shRNA and optimized AAT cDNA) via tail-vein injection. Two weeks later, AAT expression in the blood (*A*) and the transcript of piZZ (*B*) and optimized AAT (*C*) were detected in the blood and the liver, respectively. All datapoints are the mean of four mice and error bars indicate SD. shRNA-GFP: piZZ mice with the treatment of AAV8/shRNA2-GFP vector via intravenous injection; shRNA-AAT-opt: piZZ mice received AAV8/shRNA-AAT-opt vector by intravenous injection. AAT-opt: C57BL mice were treated with AAV8/shRNA-AAT-opt via intravenous injection. $*P < 0.05$ compared with pre-shRNA-treated mice and control piZZ mice without any treatments.

rescue functional AAT levels in the blood. The optimized AAT vector induced a higher AAT expression than wild-type AAT. This optimized AAT transcript also escaped the degradation of siRNA-AAT. Systemic coadministration of AAV8/shRNA2 and AAV9/AAT-opt maintained the AAT level in the blood yet silenced over 95% of piZZ AAT mRNA. Finally, injection of a single AAV8 vector encoding both shRNA and AAT-opt also induced AAT expression in the blood and piZZ AAT transcript suppression in the liver of piZZ mice.

AAV has been shown to be a very promising gene-therapy vehicle for many diseases, especially for genetic diseases, such as hemophilia and AAT deficiency. An exciting result came from a recent clinical trial in patients with Leber's congenital amaurosis. After subretinal delivery of AAV2 encoding RP65, the patients regained vision (10). AAV has also been used in two clinical trials in patients with AAT deficiency via muscular delivery (3, 4). These and other clinical trials have demonstrated AAV's safety. In recent years, great progress has been made for AAV as a gene-therapy vector, including more AAV serotypes and variants isolated as gene-delivery vehicles and generation of scAAV vector to enhance AAV transduction (6, 7).

Some patients with AAT deficiency, specifically the piZZ phenotype, have liver disease resulting from accumulation of misfolded mutant AAT protein in the liver cells. Although much effort has been spent on increasing AAT levels by gene therapy, including utilization of naked DNA and viral vectors to target the liver or other tissue, these approaches only increase AAT level in the blood to protect lung damage but do not prevent liver disease development and progression. For prevention of liver diseases caused by piZZ protein accumulation, several drugs have been explored to inhibit protein polymerization and decrease liver injury, as well as increase the mutant protein secretion. However, these agents only have transient effects. Gene therapy may represent a very promising approach to treat the liver diseases via decrease in synthesis or correction of the mutant protein. After administration of scAAV8/shRNA2 in piZZ mice, an efficient knockdown (>95%) of AAT expression was observed in this study. These results indicate that nearly all hepatocytes are transduced by scAAV8/shRNA2 vector. The silencing efficacy of the piZZ transcript presented here using the scAAV8 vector and a single shRNA is greater than that found using SV40 vector-delivery of a ribozyme (50% reduction of AAT expression), and an improvement over the knockdown effect observed in a previous study in which single-stranded AAV8 was used to deliver three shRNAs (11, 12). This result may be caused by the improvement of scAAV over traditional ssAAV and lower dosage of particles used in the previous AAV study. Because the shRNAs used for this study target regions shared by both wild-type AAT and piZZ AAT transcripts, the shRNA2 will function for all piZZ genotypes.

In this study we also provided three approaches to restore the concentration of AAT in the blood to protect lung damage. The first approach is to use the muscle-tropic AAV vector (AAV6) to deliver AAT into muscular tissues. Although the AAT level was rescued, this approach still has disadvantages. For example, systemic administration of AAV8/shRNA may also transduce muscles to degrade the AAT transcript after AAV6/AAT intramuscular injection. In addition, AAV transduction efficiency is usually lower in muscles than in the liver. Utilization of an optimized AAT construct for intramuscular injection can partially address the first concern. The second approach was to systemically apply a scAAV8/shRNA vector followed by an AAV9/AAT-opt vector. Again, there may be some valid concerns on this application, as we don't currently know the distinct mechanisms of AAV transduction between different serotypes: for example, there is a possibility of shared trafficking among serotypes even though lower neutralizing antibody cross-reactivity has been demonstrated among AAV8 and AAV9 (7), which may result in transduction competition between virions within the same cell. Thus, a third strategy would be to package both shRNA and AAT-opt cassettes into a single AAV8 virion. Because of the size limitation of AAV payload, we could not encapsidize the dual gene cassette into a self-complementary format, which would improve transduction efficiency and require a lower dose of vector to achieve the similar silence effect as scAAV virus. It is possible that two promoters in one construct may destabilize transgene expression from shRNA or AAT-opt, although the piZZ AAT transcript was knocked down and simultaneously AAT-opt was detected after injection of AAV8/shRNA-AAT/opt vector in our experiment (Fig. 4), supporting the activity of both promoters of one vector backbone.

Although no acute side effects related with AAV administration in clinical trials were reported, one concern was raised about the potential risk of capsid-specific cytotoxic T-lymphocyte (CTL)-mediated elimination of AAV transduced target cells (13). Although a capsid-specific CTL response can be elicited via both classic class I antigen presentation and cross-presentation pathways, the concept of capsid-specific CTL-mediated elimination of AAV-transduced target cells has not been reproduced in mouse models (14). One interpretation for the discrepancy between human and mouse is that transgene tolerance has been demonstrated by AAV vector targeting the liver in mice. Recent studies have shown that the liver tolerance can be reversed by up-regulation of inflammatory signals (15). Accumulation of

piZZ misfolded protein in the endoplasmic reticulum can specifically activate autophagy (16), and autophagy activation is associated with an inflammatory response (17). Future studies should address whether capsid-specific CTLs are able to eliminate AAV-transduced hepatocytes and lead to therapeutic failure in mice with the piZZ mutation.

Overexpression of shRNA delivered by gene-therapy vectors can induce cytotoxicity, resulting in organ failure and fatality in animal models because of oversaturation of endogenous cellular RNAi factors (18). No mice in the present studies died of the shRNA treatment, perhaps because of the low dose of shRNA vector and the weak H1 promoter used. In summary, our results demonstrated that an AAV vector can be used to deliver AAT shRNA to the liver and efficiently knock down the AAT transcript to prevent liver diseases. Utilization of intramuscular injection of AAV6/AAT and coadministration of AAV9/AAT-opt with AAV8/shRNA, as well as delivery of a single AAV8 vector encoding both shRNA and AAT-opt, could restore AAT expression in the blood to prevent liver disease development and treat low-AAT related lung diseases. The approach presented here has potential application to other diseases associated with misfolded proteins, such as Huntington disease and amyotrophic lateral sclerosis.

Materials and Methods

Generation of AAV Constructs. At first, the self-complementary shRNA AAV vector (pSC-H1–shRNA-empty) was constructed by swapping of fragment from pSUPER.retro.puro (OligoEngine) into pSC-TTRmvmFIX digested with endonucleases EcoRI and BsrGI (9), in which shRNA was driven by the RNA Poly III H1 promoter. The annealing of pairing oligos (Table S1) generated a BamHI terminus on the 5′ end and a Hind III terminus on 3′ end, which was cloned into the dsAAV/siRNA-empty backbone digested with BglII (the correct clone loses BglII site) and HindIII to form constructs pSC-shRNA1, pSC-shRNA2, and pSC-shRNA3. Codon optimization of human AAT cDNA specific for mouse tissues was designed and synthesized by Geneart. After digestion with EcoRI and NotI, the AAT-opt fragment was used to substitute the AAT fragment of pTR/CBA-AAT, generating the clone pTR/CBA-AAT-opt. To clone the pTR-shRNA-AAT/opt construct, the fragment from pSC-shRNA-AAT2 by the digestion of EcoRI (blunt) and BsrGI was inserted into pTR/CBA-AAT cut by BglII (blunt) and BsrGI. The clone pTR-shRNA-GFP was obtained by swapping the AAT-opt fragment in pTR-shRNA-AAT-opt with digestion of EcoRI (blunt) and NotI by the GFP fragment in pTR-eGFP with digestion of AgeI (blunt) and NotI. pTR-CBA-AAT-piZZ was generated by introduction of the piZZ mutation into the pTR/CBA-AAT plasmid.

Animal Experiments. C57BL mice and BALB/c were purchased from the Jackson Laboratory. The piZZ transgenic mice have been described before (12) (generously provided by Terence R. Flotte, University of Massachusetts Medical School, Worcester, MA). All mice were maintained in a specific pathogen-free facility at the University of North Carolina at Chapel Hill. All procedures were approved by the University of North Carolina Institutional Animal Care and Use Committee.

ELISA for Detection of AAT. A 96-well plate was coated with rabbit anti-human AAT antibody (Sigma) at 10 μg/mL overnight at 4 °C. After blocking with the sample dilution solution (PBS with 2.5% bovine albumin and 0.05% Tween-20) for 1 h at room temperature, diluted serum in 100 μL total was added to the well and incubated at room temperature for 2 h. After washing four times with PBS, 100 μL of HRP-conjugated goat anti-human AAT antibody (Abcam) (10 μg/mL) was added for 1 h at room temperature. After further washing, the color was developed by addition of the TMB substrate (Pierce) and arrested by 10% H_2SO_4. Optical density was read with an ELISA plate reader. Murine AAT was nondetectable with this assay.

Statistical Analysis. Statistics were performed using the Student *t* test. A *P* value of less than 0.05 was considered a significant difference and is marked in the figures with an asterisk.

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